University of Alberta

The Function of Plasmid O157 on *Escherichia coli* O157 Binding to Tissue Culture Cells

by

Zhuolin Han

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Medical Microbiology and Immunology

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ABSTRACT

All Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, and many non-O157 EHEC serogroups, possess a large virulence-associated plasmid. In *E. coli* O157:H7, the virulence-associated plasmid is called pO157. I found that localized adherence of a plasmid-cured EHEC O157:H$^-$ strain (E32511) to human laryngeal epithelial (HEp-2) cells and human embryonic Intestine 407 cells was significantly reduced in comparison to that of the wild-type O157:H$^-$ strain harboring the plasmid. In contrast, both the wild-type and the plasmid-cured O157:H$^-$ derivatives bound to the same level to human colon carcinoma (T84) cells and human colon adenocarcinoma (Caco-2) cells. Neither the pO157-dependent *E. coli* O157:H$^-$ binding to HEp-2 or Intestine 407 cells nor the pO157-independent binding to T84 or Caco-2 cells was affected by the presence of mannose. These results indicate that *E. coli* O157:H$^-$ (E32511) expresses at least two type 1 pili-independent (mannose resistant) tissue culture cell attachment phenotypes, one of which requires the presence of the virulence-associated plasmid.

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LIST OF ABBREVIATIONS

AA	aggregative adherence
A/E	attaching and effacing
ATCC	the American Type Culture Collection
BFP	bundle-forming pili
BSA	bovine serum albumin
Caco-2	human colon adenocarcinoma
CDC	US Centers for Disease Control and Prevention
DA	diffuse adherence
eae	*E. coli* attaching and effacing
EHEC	enterohemorrhagic *Escherichia coli*
EPEC	enteropathogenic Escherichia coli
esp	*E. coli* secreted proteins
FBS	fetal bovine serum
HCT-8	human ileocecal epithelial
HeLa	human cervix epithelial carcinoma
HEp-2	human larynx epithelial
Hly	hemolysin
HRMEC	human renal microvascular endothelial cells
HUS	hemolytic uremic syndrome
HUVEC	human umbilical vein endothelia cells
LA	localized adherence
LB	Luria broth

LEE	the locus of enterocyte effacement
IL-1β	interleukin 1β
Intestine 407	human embryonic intestine
LPS	lipopolysaccharides
MEM	minimal essential medium
ORFs	open reading frames
PA	peripheral adherence
PBS	phosphate-buffered saline
SMAC	Sorbital MacConkey agar
Stx	Shiga toxin
T84	human colon carcinoma
Tir	translocated intimin receptor
TNF- α	tumor necrosis factor α
TSB	tryptic soy broth
TTBS	Tris-buffered salt solution with Tween
VT	Vero toxin

CHAPTER 1

General Introduction

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is an "emerging pathogen". In 1983, investigators began to realize that EHEC was a distinctive pathogen from two key epidemiological observations. Riley *et al.* [1] first reported two outbreaks of distinctive gastrointestinal disease characterized by severe crampy abdominal pain, watery diarrhea followed by bloody diarrhea, and little or no fever. Stool samples from those patients revealed a rarely isolated serotype, *E. coli* O157:H7. Then Karmali *et al.* [2] concluded that there was a causal link between *E. coli* O157:H7 infection and hemolytic-uremic syndrome (HUS). These *E. coli* produced a cytotoxin, which was toxic to African Green Monkey Kidney (Vero) Cells [3]. Histopathologically, these *E. coli* strains cause attaching and effacing (A/E) lesions on epithelial cells [4]. A/E lesions are characterized by localized destruction of the apical microvilli of intestine epithelial cells resulting from the rearrangement of the cytoskeleton and intimate attachment of the bacteria [5]. Most of these *E. coli* strains contain a 60-MDa plasmid [4]. Thus, the discoveries led to the recognition of a novel pathogen, EHEC, the group of *E. coli* that expresses Shiga toxin (Stx), causes attaching and effacing (A/E) lesions on epithelial cells, and possess a 60-MDa plasmid [4]. Organisms of the O157 serogroup, including O157:H7 and O157:H$^-$[6] [7], have caused most outbreaks of EHEC infection. The most common non-O157 serotypes associated with EHEC disease include O26:H11, O103:H2, O111:NM, and O113:H21 [4].

1.1. Epidemiology

EHEC infection represents a significant health problem in Canada. Sporadic infections due to *E. coli* O157:H7 seem to be more common in Canada than in the United States [8] [9]. In 1991, the reported annual incidence of infection in Canada reached a maximum of 5.3 cases per 100,000 inhabitants and by 1993 had decreased to 3.0 per 100,000. More recently, the reported incidence has again increased [9]. The Laboratory Center for Disease Control in Canada has also been receiving information about some outbreaks. Sixteen outbreaks were reported. Nine of these were family outbreaks (19 cases), two were restaurant outbreaks (10 cases), three were institutional outbreaks (21 cases), one was a day care outbreak (9 cases), and one was a waterborne outbreak [9].

On the other hand, The Centers for Disease Control and Prevention (CDC) estimates the annual disease caused by O157:H7 alone in the United States is more than 20,000 infections and 250 deaths. The failure of some clinical laboratories to screen for this organism means that these numbers may be even greater than reported [10]. The results from CDC in 1996 showed national incidence of *Campylobacter* spp. was 25 cases per 100,000 population, *Salmonella* spp. was 16 cases per 100,000, *Shigella* spp. was 9 cases per 100,000, *E. coli* O157:H7 was 3 cases per 100,000, *Yersinia* spp. was 1 case per 100,000, *Listeria* spp. was 0.5 case per 100,000, and *Vibrio* spp, was 0.2 case per 100,000. O157:H7 strains represent the most common infectious cause of bloody diarrhea in the United States. Besides, HUS caused by O157:H7 infection is the most common reason of acute renal failure in children in the United States [11]. In

addition to the importance of EHEC in North America, it is also an important pathogen in Europe and Japan. In some countries of the southern hemisphere such as Argentina, Australia, Chile, and South Africa, non-O157 EHEC serotypes are more important than O157:H7 serotypes.

The animal reservoir of EHEC is large, including cattle, sheep, goats, pigs, cats, dogs, chickens, and gulls. The most important animal reservoir for EHEC is cattle [4]. Around 4% of cattle are positive for O157 at any given time [12]. More than 100 outbreaks of *E. coli* O157 infection have been reported in the United States alone since 1997. Fifty-two percent of the outbreaks were linked to consumption of beef, 16% from person-to-person spread, 14% from contaminated fruit, 12% from contaminated water, and 5% from miscellaneous foods [9].

Transmission of EHEC is linked to the consumption of undercooked ground beef, contaminated water, vegetables or unpasteurized milk [13]. Beef may become contaminated during the slaughter process, and the process of grinding beef may transfer the bacteria from the outer surface to the inner part of the ground beef so that they can survive the heat of cooking. In the United States, ingestion of undercooked hamburgers has been a main cause of outbreaks [4]. Therefore, EHEC disease sometimes is called "hamburger disease". Some infections were caused by cross-contamination with raw beef. The O157: H7 strains have the ability to grow in foods with low pH and have been found in apple juice and fermented hard salami [4]. The infectious dose for EHEC is very low. Around 100 to 200 of organisms will cause disease [4]. The organisms can persist in manure,

water troughs, and other places on farms [14]. In humans, O157 may be shed in the stool for several weeks following resolution of symptoms [14].

1.2. Clinical aspects

EHEC denotes strains that cause hemorrhagic colitis and, occasionally HUS, especially in young children and the elderly. The incubation period for EHEC diarrhea is 3 to 4 days. Patients experience non-bloody diarrhea, sometimes associated with vomiting. Bloody diarrhea follows within 1 or 2 days. The amount of blood varies from a few small streaks to stools that are almost entirely composed of blood [14]. Only 30% of the patients experience low grade fever. Most patients with hemorrhagic colitis will recover in 7 days. However, 10% of young patients, under the age of 10, will develop HUS [4]. The complications associated with EHEC infections are largely related to thrombotic microangiopathy development in a number of sites in the body. This is especially prevalent in the kidney and results in HUS. Renal histopathological studies in humans with HUS have shown changes in glomeruli that range from cortical or tubular necrosis to extensive arteriolar microangiopathy with tubular necrosis [17]. Other infected organs are the central nervous system and pancreas. The thrombotic microangiopathy is due to the action of Stx on endothelial cells lining the microvasculature in the target sites [15].

The triad of acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia characterizes HUS. Around 3~5% of HUS patients die and the same amount develop end-stage renal disease [14]. Many patients who regain renal function have chronic proteinuria, and some develop end-stage renal disease

years or even decades later. Other long-term sequelae include cholelithiasis, colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment [14]. Acute neurological complications such as stroke, seizure, and coma develop in 25% of patients. Rare complications include pancreatitis, diabetes mellitus, and pleural and pericardial effusions. Occasionally, patients will develop thrombotic thrombocytopenic purpura, a condition similar to HUS but with more prominent neurological findings and less renal involvement. [14]. Initial laboratory studies of EHEC disease are generally not revealing. Serum-leukocyte counts are elevated but the faecal leukocyte counts are less than ten per high-powered field. Endoscopies can show edematous and hyperemic lesion in the colonic mucosa, sometimes associated with superficial ulcerations or pseudomembranes [14]. The histopathological changes associated with hemorrhagic colitis in the intestine caused by O157:H7 show hemorrhage and edema in the lamina propria. Colonic biopsy specimens from many patients showed focal necrosis and infiltration of neutrophils [16].

Treatment for EHEC is presently limited to supportive care. There are some controversies about whether or not to use antibiotics to treat EHEC diarrhea. Some reports suggested that use of antibiotics would increase the risk of HUS. Bacteria lysed by antibiotics may release more toxin; and antibiotics may kill other intracolonic bacteria, causing less competition for EHEC. Others reported that early use of antibiotics could decrease the incidence of HUS [4]. A promising biological therapy using Synsorb-pk is under phase III clinical trials. Synsorb-pk

was designed to absorb Stx in the intestine when taken orally and prevents the development of HUS [18].

There are presently no vaccines available to prevent development of EHEC disease. Experimental approaches are now being developed in animals. Two vaccine options are available: livestock vaccine or human vaccine. Most of the experiments use Stx as the vaccine antigen and Stx has been proven to be protective in rabbits and pigs [4] [19]. Other candidates may involve intimin, an important adhesin for EHEC [20] or lipopolysaccharides (LPS) [21].

1.3. Diagnosis

Most strains of O157:H7 do not ferment D-sorbitol within 24 hours. Sorbital MacConkey agar (SMAC), therefore, is used as selective screening medium. Sorbital-nonfermenting strains should be tested as potential O157:H7. However, some non-O157 and O157:H$^-$ ferment sorbital, which makes the SMAC test not very useful in detecting non-O157 strains and O157:H$^-$ strains. Other experiments were designed for detecting O or H antigens, Stx, eae, EHEC-*hyl* and other structures of EHEC [4, 13]. The experimental methods range from immunological assays to gene level like PCR. PCR analysis of primary fecal cultures is probably the most sensitive and specific means of screening for the presence of EHEC [22].

1.4. Pathogenesis

1.4.1. Plasmids and Pili

Pathogenic mechanisms in EHEC are not completely understood. All O157 *E. coli*, and many non-O157 EHEC serogroups, harbor a large virulence-associated plasmid. The large plasmids in O157 strains are called pO157. The complete

DNA sequence of the pO157 from EHEC strain O157:H7 EDL 933 was determined and some virulence genes, like a 2997-bp plasmid-encoded enterohemolysin (EHEC-*Hly*) were identified [23]. Hemolysin is responsible for lysing erythrocytes *in vivo* and thus may provide iron to support bacterial growth. Strains that produce hemolysin are not hemolytic on standard blood agar but produce small, turbid hemolytic zones on washed sheep RBC agar supplemented with Ca^{2+} [22]. This is in contrast to the extracellular alpha-hemolysin of *E. coli*, which produces a large, clear zone of haemolysis, detectable after 4 to 6 hours of incubation [24]. An operon containing four open reading frames encodes hemolysin and the operon has 60% homology with that of *E. coli* alpha hemolysin. The genes are named EHEC-*hlyABCD* to distinguish them from the alpha-*hly* genes [25]. Schmidt *et al.* used a PCR assay to detect EHEC-*hyl*A, the structural gene for the hemolysin. They found that all 22 O157:H7 strains and 12 of 25 EHEC of other serotypes were positive for EHEC-*hyl*A [25]. All non-EHEC strains were negative. Hemolysin is associated with the virulence of *E. coli* O157 since HylA-specific immunoglobulin G (IgG) can be detected in the sera from patients recovering from HUS [24].

Other than production of the hemolysin, the role of pO157 in the pathogenesis of disease caused by O157 is unknown. There are conflicting results on the role of the plasmid in binding to tissue culture cells [4]. Karch *et al.* first reported that the plasmid was involved in bacterial binding to epithelial cells because the plasmid-cured strain bound less than the wild type to Intestine 407 cells. In addition, Karch *et al.* found that the plasmid-cured strain did not express pili, while the wild type

did. So he argued that the pO157 was necessary for synthesis of intact pili and the pili were important in binding to Intestine 407 cells [26]. Toth *et al.* reported that the plasmid-cured strain showed reduced adherence to Intestine 407 and HEp-2 cells while both wild type and its plasmidless derivative were piliated [27]. However, Fratamico *et al.* mentioned in his paper that both the plasmid-cured and parental strains adhered equally well to HEp-2 and Intestine 407 cells and both stains produced pili [28]. In contrast, Junkins *et al.* found that the plasmidless derivative bound three times better than the parental O157:H7 strain to Intestine 407 cells [29].

Besides pO157, other plasmids ranging in size from 2 to 87kb have been found in strains of O157:H7. There is no correlation of these additional plasmids with clinical disease [4]. Two of the additional plasmids, of 6.7 and 3.3 kb in size respectively, are found in up to 62% of *E. coli* O157:H7 strains [24].

1.4.2. Adhesion and Invasion

EHEC can adhere to many tissue culture cell types. In fact, one of the most useful phenotypic assays to distinguish diarrheagenic *E. coli* is the HEp-2 binding assay. There are three typical binding patterns of HEp-2 adherence, localized adherence (LA), aggregative adherence (AA), and diffuse adherence (DA) [4]. In my experiment, I found another new adherence pattern and called it peripheral adherence (PA) (Figure 1.1). However, there are arguments whether EHEC can invade tissue culture cells. O157:H7 was reported to be able to invade T24 bladder cells and HCT-8 (human ileocecal epithelial) cells. However, the level of uptake was much lower than that of enteropathogenic *Escherichia coli* (EPEC), a

major cause of non-bloody diarrhea in infants, or *Shigella flexneri* strains and was the same as that of commensal *E. coli* strains. The clinical relevance of this property is, therefore, questionable [22].

1.4.3. Shiga Toxin

The most serious problem caused by EHEC is HUS, which can be lethal. HUS is caused by the Stx, or Vero toxin (VT) EHEC produces. Stx or VT was also called Shiga-like toxin (SLT) because the toxin is related to the Shiga toxin from *Shigella dysenteria* type 1. Stx is encoded on a bacteria phage inserted into the chromosome of the organisms [30]. There are two kinds of Vero toxin produced by EHEC, Stx1 (VT1) and Stx2 (VT2). A single EHEC isolate may produce Stx1, Stx2 or both. Stx1 is identical to the Shiga toxin from *S. dysenteriae*, while Stx2 has only 56% homology with Stx1 [30]. Stx1 is highly conserved, while Stx2 displays sequence variation. The different variants are Stx2c, Stx2v, Stx2vhb, Stx2e, etc. [4].

Stx is an A-B toxin (Figure 1.2.). The A subunit has an RNA-glycohydrolase activity and the five B subunits contain the toxin's receptor binding domains [30]. The A subunit (32kDa) inhibits eukaryotic protein synthesis by catalytically inactivating elongation factor 1 (EF-1)-dependent aminoacyl tRNA binding and preventing peptide elongation [17]. An A_1 and an A_2 portion are generated when the A subunit is treated with trypsin and reduced. The A_1 portion is responsible for the glycohydrolase activity and the A_2 portion is responsible for binding the A_1 subunit to the B pentamer [11]. The B pentamer binds the toxin to a specific glycolipid receptor on eukaryotic cells, globotriaosylceramide or Gb_3 [31]. The

Figure 1.1 Schematic representation of diarrheagenic E. coli adherence phenotypes to tissue culture cells.

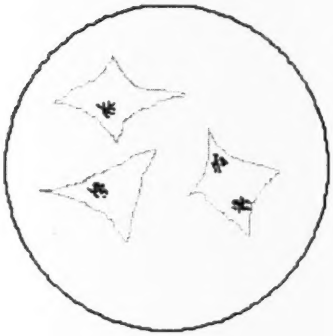
LA (localized adherence): microcolonies of bacteria attach to tissue culture cells only.

DA (diffuse adherence): even distribution of bacteria over tissue culture cells only.

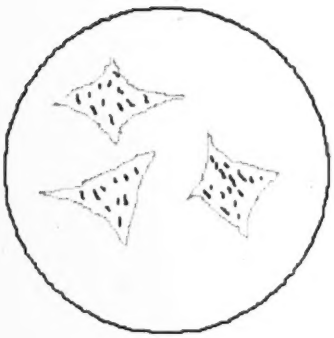
AA (aggregative adherence): clusters of bacteria attach both to tissue culture cells and glass cover.

PA (peripheral adherence): bacteria attach to the periphery of tissue culture cells.

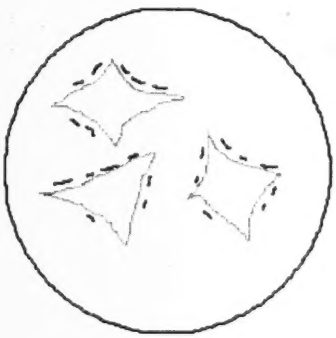
LA



DA



AA



PA

Figure 1.2. Crystal structure of Stx. The A subunit is shown in red above the five B subunits. The disulfide bridge linking A1 and A2 and the five disulfide bridges, one in each B subunit, are represented by yellow bonds connecting the α-carbons and the sulphur atoms [34].

exception is Stx2e, which recognizes globotetraosylceramide or Gb_4 [32]. Stx2e is mostly associated with pig edema disease rather than human disease. Following binding to host cell receptors, the Stx is internalized and then moves to the Golgi apparatus and then to the endoplasmic reticulum, where it interacts with the 28S rRNA subunit of the 60S ribosomal subunit [15].

Stx is essential in the development of bloody diarrhea and hemorrhagic colitis. Stx is also assumed to enter the circulation to cause HUS, although no toxin has been detected in the blood of HUS patients [33]. This may be due to the difficulty of sampling at the right time, the problem of detecting low concentrations of Stx, or the fact that Stx may bind tightly to cellular components like the erythrocytes [15]. The base level Gb_3 expression in cultured human renal microvascular endothelial cells (HRMEC) is 50 times greater than that in human umbilical vein endothelial cells (HUVEC) [17]. This may explain why Stx is more likely to target the kidney. Damage of the endothelial cells mediated by Stx may trigger platelet and fibrin deposition, leading to injury of passing erythrocytes (haemolysis) and occlusion of renal microvasculature (renal failure). Platelets are trapped in involved organs and are removed by the liver and spleen, leading to thrombocytopenia and microangiopathic hemolytic anemia[14].

Stx2 is much more toxic than Stx1 [35]. Stx2-containing strains have been reported to cause a higher incidence of HUS than strains containing Stx1 alone or both toxins [36]. Stx2 is about 1000-fold more toxic than Stx1 to renal microvascular endothelial cells [37]. Moreover, The LD_{50} of purified Stx2 for

parenterally inoculated adult CD-1 mice is 1ng, compared with 400ng for Stx1 [38].

Why Stx2 is more toxic than Stx1 is unknown. One possible explanation is that Stx2 binds less avidly to receptors than Stx1. So Stx2 can be spread through the intestine epithelial cells more efficiently [38]. Another explanation may be that the A_2 peptide of Stx1 is 4 amino acids shorter than that of Stx2. This difference may somehow affect interactions between the A_2 portion and B pentamer and thus affect the efficacy of intracellular trafficking [11]. Stx1 production can be inhibited by high concentrations of iron while Stx2 cannot. This may also contribute to the difference in toxicity between Stx2 subgroups [11].

Primary human vascular endothelial cells, pre- or co-treated with proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), or interleukin 1β (IL-1β), were more sensitive to purified Stxs [17]. Radiolabeled toxin binding studies and thin-layer chromatography techniques showed that TNF-α- and IL-1β-mediated endothelial cell sensitization to Stx is associated with the upregulated expression of the toxin binding glycolipid Gb_3 [17]. These observations may indicate that the host cytokine response to the toxins may participate in the pathogenesis of HUS by rendering vascular endothelial cells in intestinal, renal and central nervous system tissues more sensitive to the cytotoxic action of the toxins [17]. Endotoxins may be released into the circulation from the gut following intestinal damage caused by EHEC and participate in the development of vascular lesion by triggering TNF-α and IL-1β production [17].

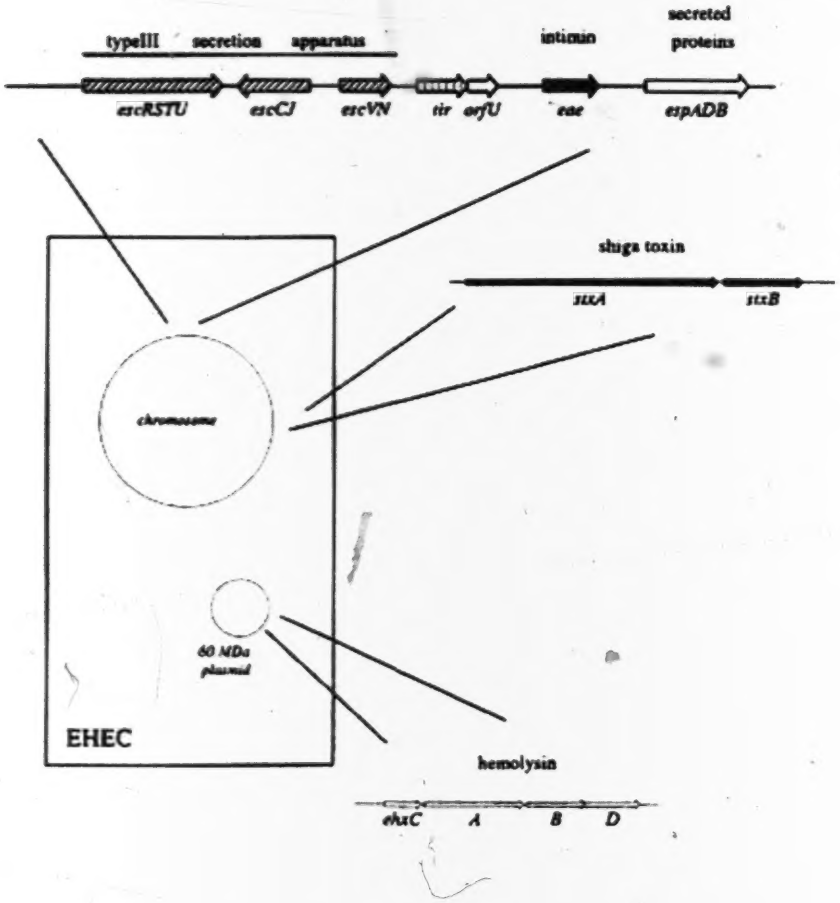
Preliminary data suggest that Stx may cause interlukin-8 (IL-8) to be secreted from intestinal epithelial cells. This could result in neutrophil migration into the underlying tissues, which contributes to the inflammatory bowel disease [15]. In a rabbit model of infection with O157:H7, the infiltration of neutrophils was blocked by the addition of antibodies to the leukocyte adhesion molecule CD18. As a result, reduction, but not elimination, of diarrhea was seen in this animal model [39].

1.4.4. LEE Pathogenicity Island

EPEC and EHEC strains have a pathogenicity island (35kb) on the chromosome called locus of enterocyte effacement (LEE), which is responsible for the attaching and effacing (A/E) lesions seen in tissue culture cells infected with EHEC and EPEC [40]. A/E lesions include loss of enterocyte microvilli and intimate attachment of the bacteria to the cell surface. Beneath the adherent bacteria, cytoskeletal components accumulate, resulting in the formation of "pedestals". This cytoskeletal change can been seen by electron microscopy and by fluorescence microscopy after staining with fluorescein-labeled phallotoxin [41]. Phallotoxins are bicyclic peptides isolated from the deadly *Amanita phalloides* mushroom and are used to label actin. The G+C content of the LEE (38.3%) is lower than that of the chromosome (50.8%), suggesting that EHEC and EPEC gained the island from another species [40].

The LEE was first described in EPEC E2348/69 by McDaniel *et al.*[42]. The LEE from EPEC E2348/69 contains 41 open reading frames (ORFs) [43] (Figure 1.3.).

Figure 1.3. Genes involved in EHEC pathogenesis [4]. LEE and genes encoding Stx are on the chromosome. Some virulent factors, like enterohemolysin, are encoded by the 60MDa plasmid.


typeIII secretion apparatus
intimin
secreted proteins
escRSTU
escCJ
escVN
tir
orfU
eae
espADB
shiga toxin
stxA
stxB
chromosome
60 MDa plasmid
EHEC
hemolysin
ehxC
A
B
D

These genes can be classified into 3 regions. The middle region contains the *eae* (attaching and effacing) gene and *tir* (translocated intimin receptor) gene[43]. The *E. coli eae* gene on the LEE island encodes a cell surface protein, intimin, involved in the intimate interaction of the pathogen and host cells and formation of A/E lesions [44] [45]. Intimin is also expressed by EPEC, EHEC, the mouse pathogen *Citrobacter rodentium*, and various EPEC-like *E. coli* strains capable of producing diarrhea in rabbits, dogs, and pigs. The *eae* locus is not found in normal flora *E. coli*, enterotoxigenic *E. coli* or other strains that cannot produce the A/E lesion [39]. The N-terminal regions of intimin are highly conserved while the C-terminal regions vary considerably. The overall protein identity of intimin between EPEC E2348/69 and EHEC O157:H7 EDL 933 is 83%. However, the first 75% of N-terminal sequences of intimin display 94% identity while the final 25% of the C-terminal regions display only 49% identity. [46]. Tir is a 78kDa protein produced in both EHEC and EPEC and is translocated by the type III secretion pathway into eukaryotic cells, where it inserts itself into the plasma membrane. Once inserted into the host cell membrane, Tir is tyrosine-phosphorylated and the molecular weight increases to 90kDa [39]. Tir is the receptor for intimin-mediated intimate attachment and A/E lesion formation [47]. Phosphorylation of Tir can been seen with EPEC and EHEC O26:H$^-$, but not with EHEC O157:H7. However, this cannot rule out the possibility that Tir is still phosphorylated in EHEC but is not detected. This may be because the Tir protein is produced at low levels and the phosphorylation is not enough to be detected [46]. In O26:H$^-$, the Tir protein is called EspE.

Upstream of *eae* and *tir* are genes encoding the Type III secretion apparatus [43], containing the *esc* (*E. coli* secretion) [48] and *sep* (secretion of *E. coli* proteins) [49] genes. The esc genes are the most highly conserved and share 98 to 100% identity [39]. Downstream of *eae* and *tir* are genes that encode several proteins secreted by the type III secretion system. The most important proteins that are encoded by this region are EspA (25kDa), EspB (38kDa), and EspD (40kDa). [50] The type III secreted proteins EspA and EspB are needed to form A/E lesions *in vivo* [51]. EspA in EPEC is a filamentous organelle that is present on the surface of the bacteria during the early stage of A/E lesion formation. The cylindrical EspA filaments are approximately 7-nm-diameter, fimbrial-like structures [52]. EspA forms the bridge between bacteria and tissue culture cells and allows EspB [52] and Tir [47] to pass into the infected tissue culture cells. EspB secretion is cell-contact-dependent [40]. The EspB protein is responsible for triggering the signal transduction events in the host cell. The cellular changes associated with EPEC-mediated A/E lesion formation include increases in inositol phosphates and intercellular calcium, activation of protein kinase C, phosphorylation of myosin light chain and tyrosine phosphorylation of Tir. In EHEC, all signaling events but tyrosine phosphorylation of Tir are observed, with the exception of EHEC O26:H11, as mentioned above [53]. EspD is important for the formation of EspA-containing filaments and is transferred to the cytoplasm and integrated into the cytoplasmic membranes of infected cells [54]. Mutation of any one of the *espABD* genes abolishes the A/E phenotype [39]. EspC in EPEC is called EspP in EHEC O157:H7, and SspA in EHEC O26:H11. This 110kDa

protein is not encoded in the LEE, and mutation of the *espC* gene in EPEC does not affect signal transduction or A/E histopathology [16].

The secreted proteins EspA, EspB and EspD should not be confused with EspP, which is encoded on pO157. EspP is a protease capable of cleaving pepsin A and human coagulation factor V. Degradation of factor V could contribute to the mucosal hemorrhage observed in patients with hemorrhagic colitis [55]. While EHEC hemolysin genes occur in almost all the EHEC O157 strains, *espP* genes are present in only 66% of the strains and lower for non-O157 strains. The 35-kb locus of LEE in EHEC was completely sequenced recently in O157:H7 as well (Figure 1.3.) [56]. The 41 genes found in LEE of EPEC were also found in that of EHEC and were in the same order. The average nucleotide identity between EPEC E2348/69 and O157:H7 is 93.9% across the 41 shared genes [40, 43].

Although there are many similarities between EPEC and EHEC, there are many differences between them as well. Unlike EHEC, EPEC does not produce exotoxin. EPEC is invasive while EHEC is not [22]. These differences may contribute to the different diarrhea types EPEC and EHEC cause. EPEC strains have a large virulence plasmid (50~70 MDa) called the EAF plasmid that encodes bundle-forming pili (BFP), which are responsible for early binding of bacteria to tissue culture cells [4]. EHEC strains do not have the EAF plasmid and thus do not produce bundle-forming pili.

1.4.5. Other Virulence Factors

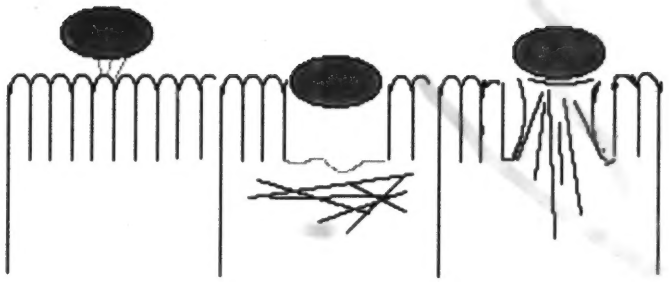
A 69-kDa outer membrane protein (OMP) encoded by the *chuA* (*E. coli* heme utilization) gene is responsible for the use of heme or hemoglobin as an ion source

during iron limited growth [57]. An 8-kDa outer membrane protein (OMP) is associated with the binding of O157:H7 to Intestine 407 cells *in vitro* and chicken caeca *in vivo*[58]. The *Iha* gene encodes a protein similar to the iron-regulated gene A (IrgA) of *Vibrio cholerae*. Iha (IrgA homologue adhesin) was found to mediate adherence of O157:H7 to HeLa and MDBK cells [59]. Lipopolysaccharide (LPS) from EHEC enhanced the cytotoxicity of Stx on human vascular endothelial cells *in vitro* but its function *in vivo* is not known [4]. Bilge *et al.* found that the O side chain of O157:H7 LPS interfered with the adherence of O157:H7 to epithelial cells [60].

1.4.6. Role of pO157 in EHEC O157 pathogenesis and objectives

The complete DNA sequence of pO157 from *E. coli* O157:H7 has been published and some potential virulence-associated genes, such as hemolysin, were identified [23]. Other than this, the role of pO157 in the pathogenesis of *E. coli* O157-mediated disease remains unclear. Due to the controversy of the role of the pO157 in adherence of the O157 strains to tissue culture cells mentioned above, I described the possible role of the plasmid and some other factors that may contribute to the adherence to the epithelial cells in this report.

Figure 1.4. EHEC Infection Model. EHEC is speculated to infect tissue culture cells in three-stage model. Following initial attachment mediated by an unknown adhesin, EHEC secretes proteins by the type III secretion system. These proteins include EspA, EspB, Tir, etc. EspB protein is responsible for triggering the signal transduction events in the host cell. Tir functions as the receptor for intimin. Then intimin mediated attachment and the following A/E lesion.



1 2 . 3

Step 1. Non-intimate attachment mediated probably by unknown adhesins, likely pili

Step 2. Signal transduction mediated by secreted protein, including EspA, EspB, and Tir, which leads to the generation of intracellular messengers and a diffuse accumulation of cytoskeleton proteins beneath the bacteria

Step 3. Intimin attachment mediated by intimin, leading to A/E lesion and pedestal formation

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CHAPTER 2

Influence of the Virulence-associated Plasmid, pO157, on Escherichia coli O157 binding to Tissue Culture Cells

2.1. Material and Methods

2.1.1. Bacterial strains and growth conditions. The bacterial strains used in this study and their descriptions are presented in Table 2.1. The *E. coli* O157:H7 (86-24) and O157:H7 CVD451 were kindly provided by Dr. B. Brett Finlay at the University of British Columbia. The *E. coli* K12 (EEU 386) and O78:H11 (H10407d) were kindly provided by Dr. M. Finlayson in the Department of Medical Microbiology and Immunology at the University of Alberta. All bacterial strains were incubated without shaking overnight at 37°C in Luria broth (LB), tryptic soy broth (TSB), or glucose-free TSB.

2.1.2. Tissue culture conditions. All tissue culture cells were obtained from the American Tissue Culture Collection (ATCC) and were grown at 37°C in a humidified atmosphere of 5% CO_2-95% air. HEp-2 (CCL-23) cells and Intestine 407 (CCL-6) cells were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS, Sigma) and MEM supplemented with 15% FBS respectively. Caco-2 (HTB-37) cells were grown in MEM supplemented with 20% FBS. T84 (CCL-248) cells were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS. Vero (CCL-81) cells were grown in MEM containing 5% FBS. To prepare sub-confluent tissue culture monolayers for the

Table 2.1. Strains used.

Serotype (Strain)	**Description**	Reference
HB101	Nalidixic acid-resistant mutant, hybrid of *E. coli* K and *E. coli* B strains, phenotypically non-piliated (negative control for binding assay, salt aggregation assay and hemagglutination assay.)	[1]
O157:H$^-$ (E32511)	Enterohemorrhagic *E. coli* O157 isolated from HUS patient, producing SLT-2 only.	[2]
O157:H$^-$ (rif^r, pO157)	The rifampin resistant derivative of E32511 produced in our lab, producing SLT-2 only.	this paper
O157:H$^-$ (rif^r, ΔpO157)	The rifampin resistant, plasmid-cured derivative of E32511 produced in our lab, producing SLT-2 only.	this paper
O78:H11 (H10407d)	Enterotoxigenic *E. coli* strain, CFA ^{a}/I $^+$ (positive control for salt aggregation assay)	
E. coli K12 (EEU 386)	Produce common type 1 pili (positive control for hemagglutination assay)	
O127:H6 (E2348/69)	Wild type enteropathogenic *E. coli*	[3]

binding assays, approximately 5 x 10^4 cells were seeded onto 12-mm diameter sterile glass cover slips in 24-well tissue culture plates. The plates were then incubated at 37°C and the resulting monolayers were used the following day.

2.1.3. Removal of pO157 from O157:H⁻ (E32511). A rifampin-resistant mutant of the parental O157:H⁻ (E32511) strain was isolated by spontaneous mutation after cultivating the organisms on Difco tryptic soy agar (TSA) plates containing 250 μg/ml rifampin. The rifampin-resistant mutant was spontaneously cured of pO157 after the conjugal introduction of a temperature-sensitive plasmid, F'114tslac::Tn5. The loss of pO157 was confirmed by PCR specific for the *hlyA* gene [6]. Conjugation was conducted at 32°C and 30 μg/ml kanamycin and 250 μg/ml rifampin were used as selective agents. The resulting strain, containing the plasmid F'114tslac::Tn5, was then grown in rifampin-supplemented medium at 37°C to eliminate the F' plasmid.

2.1.4. PCR. PCR for detection of hlyA was carried out as described previously by Schmidt *et al.* [6] This PCR method was developed to amplify a 1,551-bp fragment from the 5' region of the *hyl*A gene. The primers were hlyA1 (5'-GGT GCA GCA GAA AAA GTT GTA G-3') and hlyA4 (5'-TCT CGC CTG ATA GTG TTT GGT A-3'). The amplified DNA fragment was analyzed by electrophoreses on a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination.

2.1.5. EHEC adherence assay. Bacteria, which were grown without shaking to mid-stationary phase overnight at 37°C, were added to the sub-confluent tissue

culture monolayers at a concentration of 5×10^6 bacteria/well. The adherence medium was identical to the tissue culture growth medium with the addition of 1% (w/v) mannose. EHEC and tissue culture cells were incubated for 2.5 h at 37°C in a humidified atmosphere of 5% CO_2-95% air, washed three times with sterile phosphate-buffered physiological saline (PBS, pH 7.4), overlaid with fresh adherence medium for an additional 2.5 h, washed three times with phosphate-buffered physiological saline (PBS, pH 7.4). The monolayers were then fixed and stained with Geimsa stain for microscopic evaluation [7]. Some adherence assays, using HEp-2 cells, were done for 1 or 2.5 hours only. The PBS washing step was omitted in these assays.

2.1.6. Salt aggregation test. This test was performed essentially as described by Lindahl *et al.* [8]. The bacteria were grown at 37°C for 18 h without shaking in LB. The cultures were then centrifuged at 3000 × g for 5 min. in 1.5 ml micro-centrifuge tubes using an Eppendorf model 5415C centrifuge and the resulting bacterial cell pellets were resuspended at a concentration of 5×10^9 bacterial cells/ml in 2 mM sodium phosphate buffer (pH 6.8). Twenty five µl of this suspension was then mixed with an equal volume of $(NH_4)_2SO_4$ at different concentrations ranging from 0.2 to 4.0 M in glass depression slides. The slides were gently rocked for 2 min. at room temperature and observed for cellular aggregation against a black background.

2.1.7. Tetramethyl urea (TMU) binding inhibition assay. This assay was performed according to the procedure described earlier by Mantle *et al.* [9].

Basically, the EHEC adherence assay was performed as described above except that different concentrations of TMU were added to the medium.

2.1.8. Mannose-sensitive hemagglutination assay. Whole *E. coli* from overnight static cultures were mixed with an equal volume of washed Guinea pig erythrocytes suspended in PBS or PBS plus 1% mannose in the depressions of a porcelain tile. The suspensions were gently mixed with a clean automatic pipette tip and then the tile was rocked until noticeable hemagglutination was observed. Hemagglutination was recorded in units from 1 to 4 plus using the criteria described by Old [10].

2.1.9. Comparison of Stx produced by *E. coli* O157:H⁻ strains. The Vero cytotoxicity assay was used to compare Stx production by the different O157:H⁻ strains [11]. One ml of whole bacterial cells was treated with 0.1mg/ml polymyxin B sulfate for 30 min. at 37°C to release the toxin. Bacteria were removed by centrifugation at 16,000 × g for 5 min. in 1.5 ml micro-centrifuge tubes using an Eppendorf model 5415C centrifuge. The resulting supernatant solution was filter-sterilized through a 0.2 μm filter. 1:5 serial dilutions of the extracts were prepared and added to confluent Vero cell monolayers in 96-well microtiter plates. After incubation for 48 hours, the Vero cells were fixed with methanol and stained with Geimsa stain. The results were recorded using a microtiter plate reader set at a wavelength of 620 nm and the data were analyzed as described previously [11].

2.1.10. Isolation of pili from *E. coli* 0157:H⁻ and 0157:H⁻ (rif^r, Δp0157). Pili were isolated by a modification of the procedure described by Karch *et al.* [12].

Bacteria were grown at 37°C, without shaking, in glucose-free TSB. The bacteria were then harvested by centrifugation at 6,000 × g for 10 minutes at 4°C (Avanti J-20 centrifuge, JA-20 rotor). The bacterial pellet was subsequently resuspended in sterile ice cold PBS and blended using a Sorvall Omni-mixer for 45 seconds at a speed setting of 5. These suspensions were centrifuged at 12,000 × g for 25 minutes, the supernatant solution was adjusted to 25% saturation with solid ammonium sulfate and the solution was allowed to sit for 18 hours at 4°C. Pili, precipitated under these conditions, were harvested by centrifugation at 10,000 × g for 25 minutes. The final pellet was resuspended in PBS, dialyzed extensively to remove ammonium sulfate, and analyzed by transmission electron microscopy. Sometimes pili were further purified by applying them to a 35 ml linear CsCl gradient. This gradient was subjected to ultracentrifugation at 90,000 × g for 14 hours at 4°C. Pili at top band of CsCl gradient (density approximately 1.2g/cm^3) were removed and dialyzed in PBS over night to remove CsCl.

2.1.11. Transmission electron microscopy. 10 µl of pili suspension isolated by ammonium sulfate was placed onto a freshly prepared carbon-coated electron microscope grid. The drop was removed by blotting the edge of the grid with filter paper and then placing 10 µl of 1.0% sodium phosphotungstate onto the grid to negatively stain the pili. The drop was removed as above. Grids were allowed to air dry before viewing. Sterilized broth was examined the same way.

2.1.12. Scanning electron microscopy. The 12 mm diameter cover slips containing cells were fixed with freshly prepared 2.0% (v/v) glutaraldehyde, in phosphate buffer pH 7.3 overnight. The samples were washed three times in

phosphate buffer and further fixed in 1% (w/v) osmium tetroxide (Fisher Scientific cat. #A235) in phosphate buffer for 2 hours at 4°C. The sample was again washed in phosphate buffer, dehydrated in a graded series of ethanol at room temperature then critical point dried (Balzers Critical point dryer #CPD 030). The cover slips were placed on standard Cambridge scanning electron microscopy stubs using double faced tape, lightly sputter coated with gold then examined in a Hitachi S 4000 field emission scanning electron microscope at an accelerating potential of 2.0 kV.

2.1.13. Statistics. Results are expressed as means ± standard deviation. The Student's t test (unpaired) was used to compare differences in the data sets; $P < 0.05$ was considered significant.

2.2. Results

2.2.1. Binding assay. When grown in LB, both *E. coli* O157:H$^-$ and its plasmid-cured strain displayed the classic pattern of localized adherence (LA) when the organisms were applied to HEp-2 and Intestine 407 cells. However, the LB-grown plasmid-cured *E. coli* O157:H$^-$ strain bound significantly less well to HEp-2 and Intestine 407 cells (Figure 2.1. and Figure 2.2). Moreover, time-course HEp-2 cell binding assays, demonstrated that both the wild-type and plasmid-cured *E. coli* O157:H$^-$ initially bound to HEp-2 cells as single bacteria. These single bacteria eventually evolved into microcolonies on the surface of the HEp-2 cells (Table 2.2.). The positive control for all the binding assay is O157:H$^-$ (rif^r, pO157) and

both O157:H$^-$ and O157:H$^-$ (rif^r, pO157) bound the same to tissue culture cells I used in my experiment.

In most *E. coli* O157 adherence assays, the organisms are typically grown in either TSB or LB. However, I found that the binding of the wild-type *E. coli* O157:H$^-$ strain to HEp-2 cells was significantly reduced when the organisms were grown in TSB. Since, in contrast to LB, the TSB I used in these binding experiments contained glucose, I investigated the effect of this carbon source on the HEp-2 cell adherence phenotype of the wild-type and plasmid-cured *E. coli* O157:H$^-$ strains. When grown in glucose-free TSB, I observed a significant increase of both these strains in the binding to HEp-2 cells (Table 2.3.).

Caco-2 and T84 cells were also used in the *E. coli* O157:H$^-$ binding assays because these two tissue culture cell lines are derived from human colon cells and may better represent the natural host cells for these organisms. Wild-type and plasmid-cured *E. coli* O157:H$^-$, when grown in LB, bound to the same extent to Caco-2 and T84 cells. However, the adherence pattern of these bacteria to Caco-2 and T84 cells was different from that observed when HEp-2 and Intestine 407 cells were used in these binding assays. Both wild-type and plasmid-cured *E. coli* O157:H$^-$ displayed aggregative adherence pattern on Caco-2 cells and peripheral adherence pattern on T 84 cells respectively(Figure 2.1. and Figure 2.2). The adherence of wild-type and plasmid-cured O157:H$^-$ to Caco-2 and T84 cells was not due to type 1 pili since including 1% mannose in the medium did not inhibit this binding.

Figure 2.1. Light micrograph of HEP-2 and Caco-2 cells after incubation with O157:H$^-$ and O157:H$^-$ (rif^r, ΔpO157) grown in LB for 5 hours. A: O157:H$^-$ binding to HEp-2 cells as LA pattern. B. O157:H$^-$ (rif^r, ΔpO157) binding to HEp-2 cells. C. O157:H$^-$ binding to Caco-2 cells. D. O157:H$^-$ (rif^r, ΔpO157) binding to Caco-2 cells. Both O157:H$^-$ and O157:H$^-$ (rif^r, ΔpO157) bound to Caco-2 cells as aggregate adherence patterns.

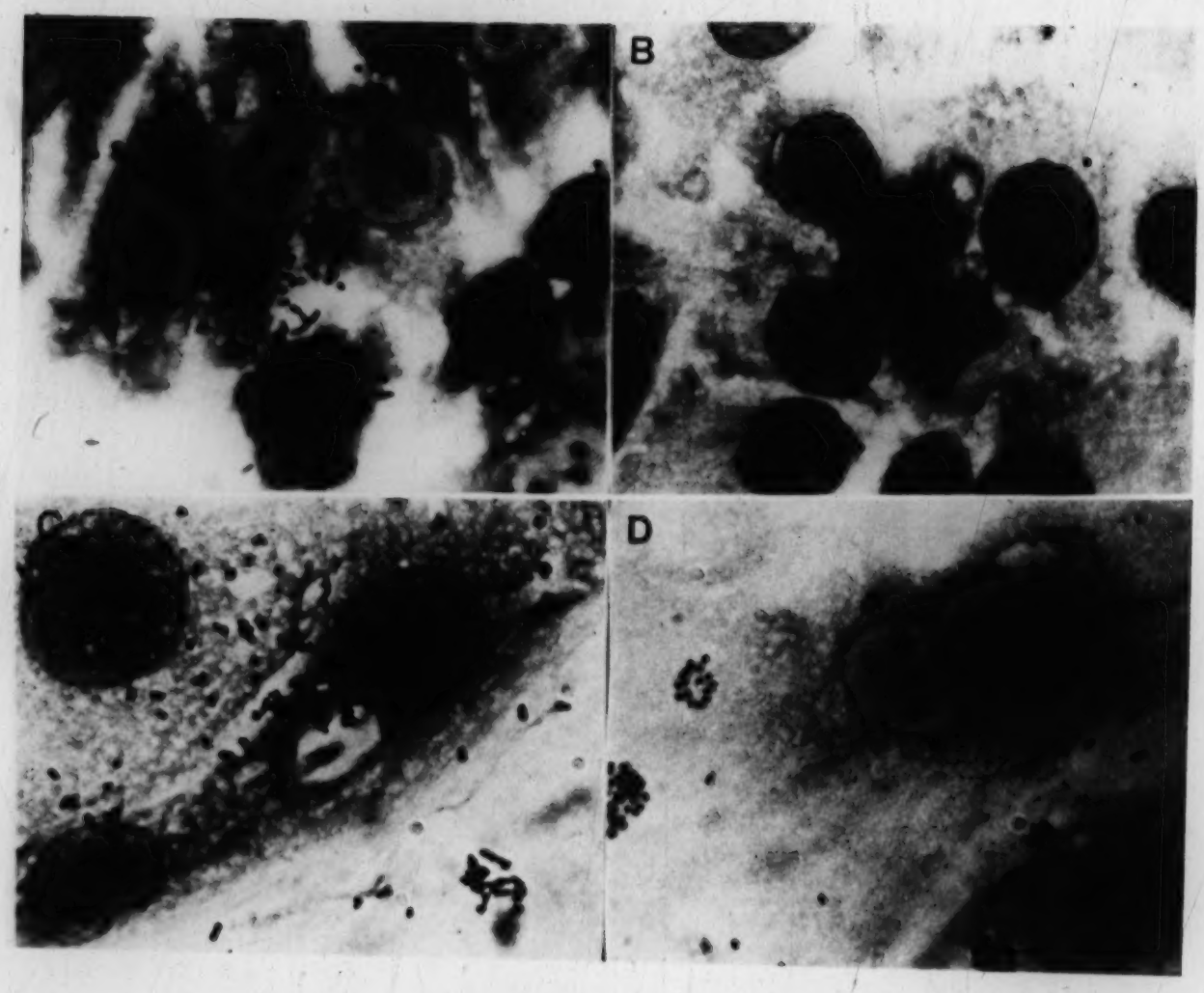

B
D

Figure 2.2. Light micrograph of Intestine 407 and T 84 cells after incubation with O157:H$^-$ and O157:H$^-$ (rif^r, ΔpO157) grown in LB for 5 hours. A: O157:H$^-$ binding to Intestine 407 cells as LA pattern. B. O157:H$^-$ (rif^r, ΔpO157) binding to Intestine 407 cells. C. O157:H$^-$ binding to T84 cells. D. O157:H$^-$ (rif^r, ΔpO157) binding to T 84 cells. Both O157:H$^-$ and O157:H$^-$ (rif^r, ΔpO157) bound to T 84 cells as peripheral adherence patterns.

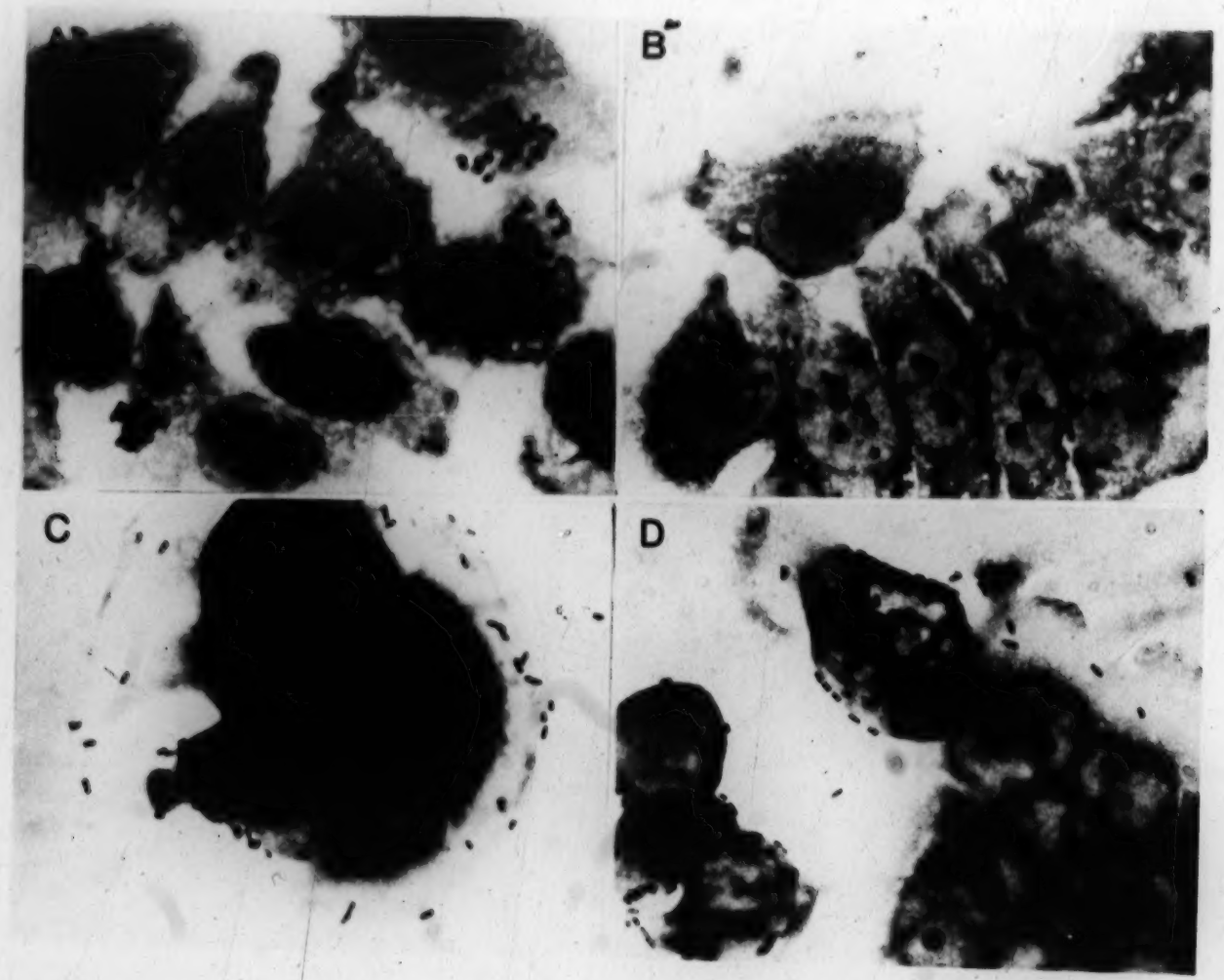

A
B
C
D

Table 2.2. Time-course HEp-2 adherence assay (% adherence)

	O157:H⁻			O157:H⁻ (rif^r, ΔpO157)		
	1 hour	2.5 hour	5 hour	1 hour	2.5 hour	5 hour
LA	0	6.7±1.5	100	0	1±1	15.5±5.4
TOTAL	5.7±4.2	98.6±1.6	100	4.3±1.9	52.8±5.9	64.2±0.71

The O157 strains were grown in Glucose-free TSB.

Total: the percentage of HEp-2 cells that have O157 bound either as LA or as one or two single bacteria.

Table 2.3. HEp-2 adherence assay with different growth media (% adherence)

	LB	TSB	Glucose-free TSB
O157:H$^-$	24.6±7.0 (n=8)	17.9±1.2 (n=4)	100±0 (n=4)
O157:H$^-$ (rif^r, ΔpO157)	2.1±1.5 (n=8)	0.8±1.0 (n=4)	15.5±5.4 (n=4)

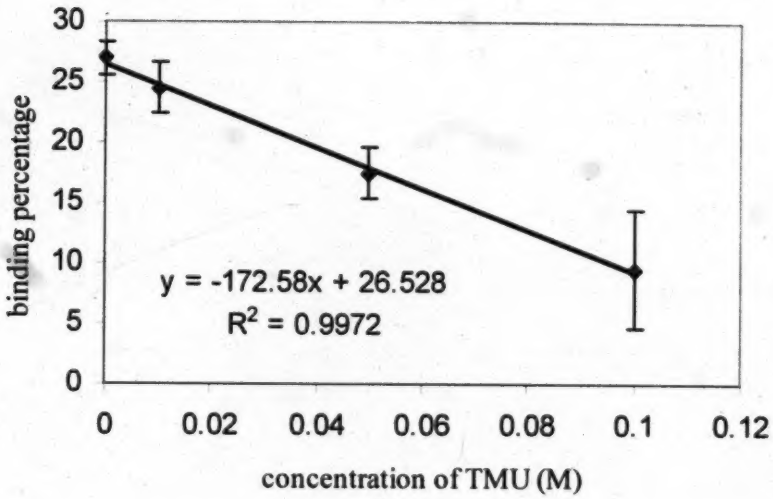
The binding time was standard 5 hours.

The percentage of binding was counted as the percentage of HEp-2 cells that have O157 bound as LA (>5 bacteria/microcolony)

Tetramethylurea (TMU) decreases hydrophobic interactions between bacteria and tissue culture cells [13]. When TMU was added to the binding medium, wild-type *E. coli* O157:H⁻ binding to HEp-2 cells decreased in a concentration-dependent manner (Figure 2.3.). TMU did not affect the viability of either the bacteria or the HEp-2 cells during the time course of the assay. This assay suggested that hydrophobic interactions might play a role in wild-type *E. coli* O157:H⁻ binding to HEp-2 cells. Accordingly, I performed the salt aggregation test [8] to exclude the possibility that the different binding phenotypes of wild-type and plasmid-cured *E. coli* O157:H⁻ in HEp-2 and Intestine 407 cells may be due to differences in hydrophobicity of the outer membranes of these two strains. Bacteria with high surface hydrophobicity aggregate in ammonium sulfate at low concentration. Using the salt aggregation test, I determined that the surface hydrophobicity of *E. coli* O157:H⁻ and the plasmid-cured strain were the same (Both aggregated in ammonium sulfate at 1.8 M). This indicated that the different HEp-2 and Intestine 407 cell binding characteristics of these two strains was not due to alterations in their surface hydrophobicity. Besides, the Vero assay confirmed that these two strains produced the same amount of Stx-2 (data not shown), which exclude the possibility that different binding of the two strains were affected by the toxin they produced.

2.2.2. Examination of *E. coli* O157:H- binding to HEp-2 cells by low voltage, high resolution scanning electron microscopy (SEM). SEM observation revealed that the bacteria appeared to bind to localized multi-microvilliated areas of the HEp-2 cells. The bacteria seemed to create depressions in the HEp-2 cell

Figure 2.3. TMU inhibition of O157:H$^-$ grown in LB binding to HEp-2 cells. Binding was calculated as the percentage of the tissue culture cells containing localized adherence *E. coli* O157:H$^-$.


30
25
20
15
10
5
0
binding percentage
y = -172.58x + 26.528
R2 = 0.9972
0
0.02
0.04
0.06
0.08
0.1
0.12
concentration of TMU (M)

surface. The microcolonies were embraced by the multi-microvilliated areas on the surface of the cells. These microcolony-associated microvilli were longer and more abundant than those protruding from regions of the cell surface to which no bacteria were bound (Figure 2.4. panels A and B). These localized multi-microvilliated areas on the HEp-2 cells were also present in the control HEp-2 cell preparation incubated without bacteria. (Figure 2.4. panel D). In addition, many of the bound bacteria produced slender surface projections, likely composed of EspA [14], which were readily distinguishable from the host cell microvilli, some of which could be seen bridging the bacterium and HEp-2 cell surface (Figure 2.4. arrows, panel C).

2.2.3. Analysis of pili. The recently sequenced *E. coli* O157:H7 EDL933 chromosome contains at least 13 genes that may be capable of encoding for proteins with putative host cell adhesive functions [15]. Those proteins include putative pili genes and outer membrane protein genes. Therefore, I wished to determine whether the enhanced adherence of *E. coli* O157:H$^-$ to HEp-2 cells when the organisms were cultured in glucose-free TSB may have been related to the production of pili from one or more of these potential adhesin genes. Transmission electron microscopy (TEM) revealed that both wild-type and plasmid-cured *E. coli* O157:H$^-$ strains produced slender (0.5 nM diameter) filamentous, pili-like appendages when the organisms were grown in glucose-free TSB (Figure 2.5.a), but not in TSB or LB. However, these pili-like appendages were also observed when I used TEM to examine sterile glucose-free TSB that had been incubated overnight at 37°C (Figure 2.5.b). Gaynor *et al.* [16] recently

Figure 2.4. SEM of O157:H$^-$ binding to HEp-2 cells. A. Low magnification showing LA. B. Higher magnification showing O157:H$^-$ were buried under the microvilli from HEp-2 cells. C Higher magnification showing the protrusion from O157:H$^-$ and one of them connecting the bacterium to the HEp-2 cell (see arrow). D. SEM of HEp-2 cells with no bacteria.

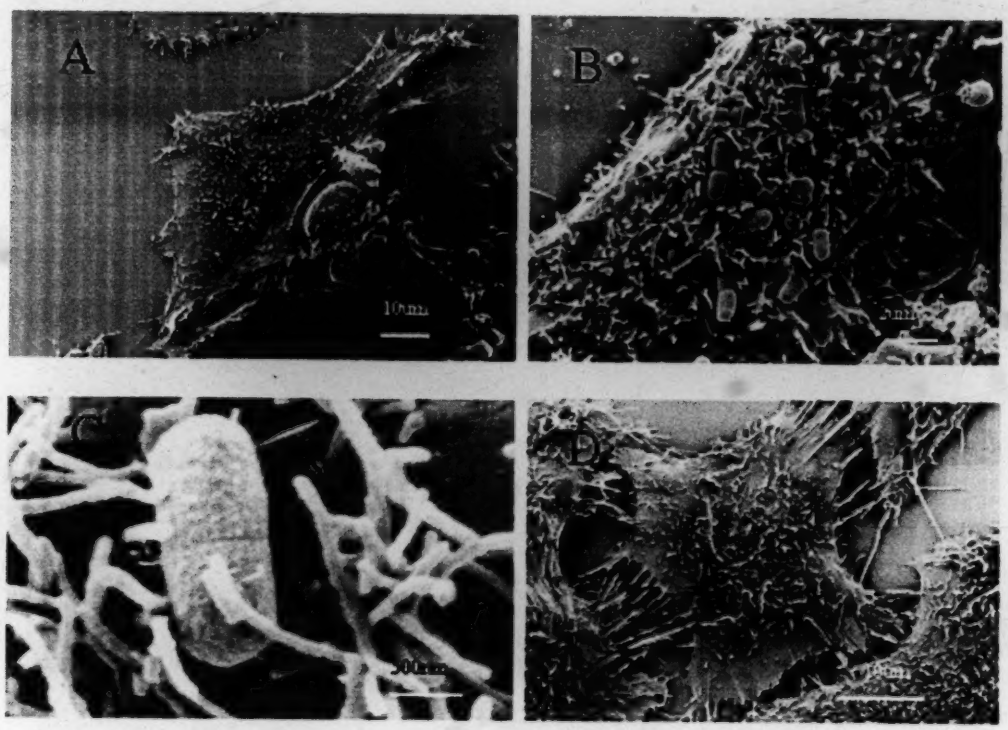
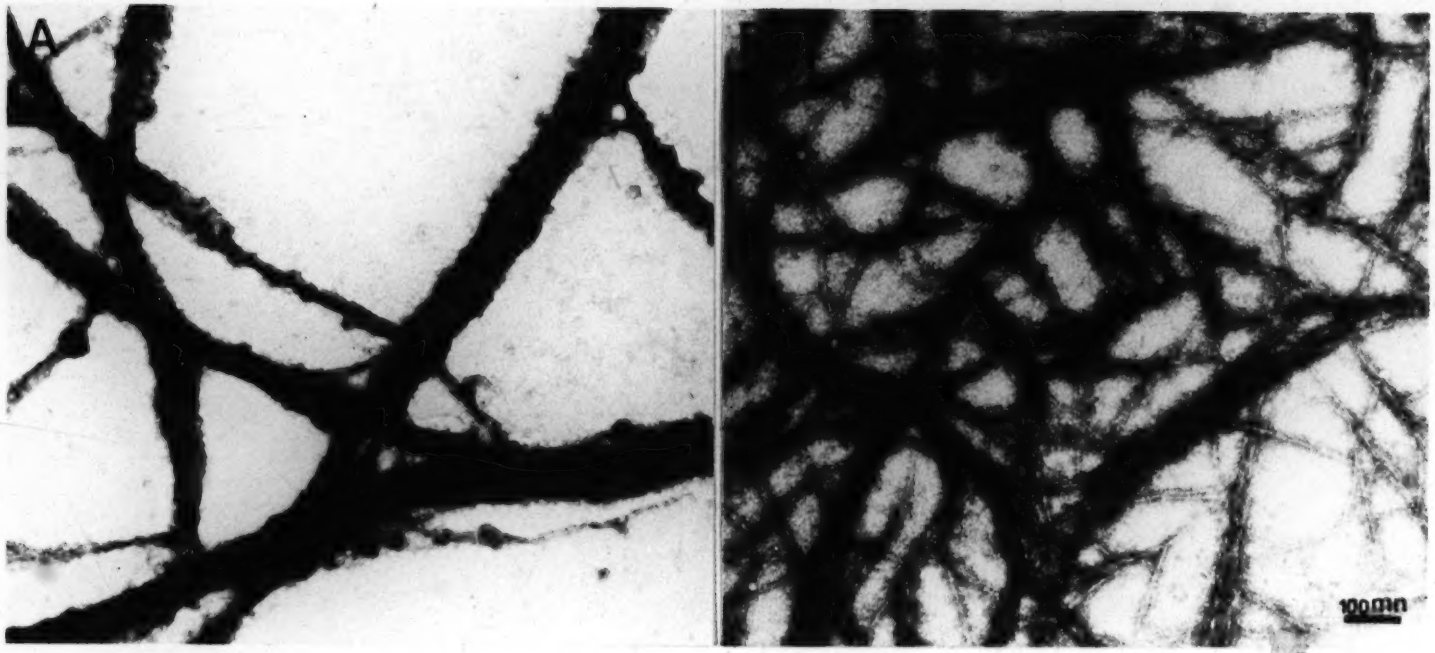

A
B
C
D

Figure 2.5. A. TEM of the pili structure precipitated by ammonium sulfate precipitation when O157:H$^-$ was grown in glucose-free TSB. The diameter of the pili structure is around 0.5 nm. B. The same pili structure was seen when sterile glucose-free TSB was incubated overnight.


A
100nm

reported the production of similar pili-like appendages at 37°C in sterile Mueller-Hinton broth supplemented with bile salts. My observation that pili-like structures were also produced in sterile glucose-free TSB suggests that this potential artifact of the culture medium may be a more generalized phenomenon than previously appreciated.

Mannose-sensitive hemagglutination assay showed that O157:H$^-$ and its plasmid-cured strains expressed mannose-resistant hemagglutination (1 plus) when they were grown in glucose-free TSB overnight but not in TSB. This indicates that O157:H$^-$ and its plasmid-cured strain may produce non-type 1 pili when grown in glucose-free TSB.

2.3. Discussion

My studies revealed that the plasmid-cured *E. coli* O157:H$^-$ strain, O157:H$^-$ (rif^r, ΔpO157), expressed an equivalent amount of Stx2 (Figure 2.7, see Appendix) and agglutinated at the same concentration of ammonium sulfate as the strain from which it was derived. These indicated that the different binding efficacy of the plasmid-cured strain and its parental strain to HEp-2 and Intestine 407 cells was due to pO157, and was not affected by the toxin or the surface hydrophobicity.

I performed binding assays using different tissue culture cell lines and obtained varied results, indicating the importance of using more than one type of cell line in these assays. The results presented here show that *E. coli* O157:H$^-$, at least, possesses two eukaryotic cell adherence mechanisms, one which depends on the presence of the virulence-associated plasmid, pO157, as exemplified by the HEp-

2 and Intestine 407 cell binding assays and a second, plasmid-independent mechanism, as demonstrated by the binding to Caco-2 and T84 cells. Many laboratories use Intestine 407 cells to represent cell line derived from the intestine. However, Intestine 407 was established via human cervix epithelial carcinoma (HeLa) cell contamination and contains HeLa cell markers [17]. Therefore, Caco-2 and T84 are more suitable human intestine derived cells. The reason different groups have reported different binding results for *E. coli* O157:H7 and its plasmid-cured derivatives may be due to strain-specific differences, differences in the culture media used, growth phase, binding times, or inoculum size. I utilized different binding media and observed different binding results with the same strain to HEp-2 cells. For example, both the parental strain and the plasmid-cured strain bound significantly more to HEp-2 cells when grown in glucose-free TSB than in TSB, suggesting that growth in glucose can inhibit O157 strains from binding to HEp-2 cells.

The sequence of pO157 did not reveal any potential adhesin genes in the plasmid [18]. So pO157 may regulate adhesins on the chromosome. There are at least 13 potential adhesin genes in the *E. coli* O157:H7 genome [15]. The *E. coli* O157:H7 adhesins which others have described include: Intimin [19], EFA1 (EHEC factor for adherence) [20], PAS (protein associated with secretion) [21], Iha (IrgA homologue adhesin) [22], and an 8-kDa outer membrane protein (OMP) [23]. However, it is unlikely that any one of these known adhesins is involved in *E. coli* O157:H7 attachment in all circumstances.

In addition to the human intestine, *E. coli* O157:H7 strains survive in the gastrointestinal tract of various animals, especially cattle [24]. Therefore, it is not surprising that EHEC may use different adherence mechanisms in different hosts. The large numbers of potential adhesins that may be encoded by the *E. coli* O157:H7 chromosome suggest that there is probably redundancy in the attachment mechanisms in *E. coli* O157:H7 strains and one or more of them may be expressed in different situations determined by different environmental factors. In addition to these specific adhesins, some non-specific factors, hydrophobic interactions for example, may also help the organisms colonize their hosts.

I discovered that both the plasmid-cured strain and its parental strain produce filamentous structures when grown in glucose-free TSB but not in TSB. The filamentous structure is not EspA since EspA is 7~8 nm in diameter [14], while the structure I found is around 0.5 nm in diameter. Although I initially felt I may have identified another fimbrial-like adhesin in *E. coli* O157:H⁻ grown in glucose-free TSB, a recent report by Gaynor *et al.* prompted us to examine the origin of the filamentous structures more carefully [16]. Gaynor *et al.* found that the pili-like structure in *Campylobacter jejuni* was an artifactual result of co-incubating broth with bile salts. My sterile glucose-free TSB incubated over night also showed the filamentous structures, which led us to consider the pili I discovered may also be an artifact.

Both EHEC and EPEC bind to HEp-2 cells as microcolonies [25]. The mechanism, however, of microcolony formation is apparently different. EPEC may bind to HEp-2 cells as pre-formed microcolonies [26], while O157:H⁻ might

bind to HEp-2 cells first as single bacteria which subsequently developed into microcolonies [27]. My binding studies support this latter conclusion that *E. coli* O157:H⁻ bind to HEp-2 cells initially as single bacteria, which subsequently develop into microcolonies. There may be two mechanisms for microcolony formation in EHEC. One is that a single bound bacterium recruits other free bacteria from the medium to form microcolonies. The other is that, during replication, the single bacterium eventually surrounds itself with daughter cells. Given their obvious entanglement in host cell microvilli (Figure 2.4. panel b), it is difficult to see how recruitment of free-floating organisms to bound bacteria contributes to *E. coli* O157:H- microcolony formation. However, it is possible that single bound bacteria could be drawn together as the result of host cell cytoskeletal action associated with the higher density of microvilli seen associated with microcolonies.

Moreover, *E. coli* O157:H⁻ binding to Caco-2 and T84 cells may be more complex, involving mechanisms which mediate localized, and aggregative adherence patterns. Identifying the adhesin or adhesins responsible for the plasmid-mediated binding of *E. coli* O157:H⁻ to HEp-2 and Intestine 407 cells will help to further reveal the complexities associated with the binding of these organisms to their host cells.

2.4. Appendix

Material and methods, supplemented results, and figures not shown in the previous sections.

2.4.1. Material and Methods

Labeling pO157 with an antibiotic selection marker. O157:H$^-$ (E32511) was conjugated with strain UB281[28], which harbored the temperature sensitive plasmid pMR5::Tn801. The conjugation was performed at 37°C on nonselective agar. The conjugated O157:H$^-$ (pO157::Tn801) was then selected on carbenicillin (250ug/ml) TSA plates incubated at 42°C.

Isolation of pO157::Tn801. O157:H$^-$ (pO157::Tn801) was grown overnight in LB with carbenicillin (250ug/ml). The plasmid was isolated according to the method of Birnbiom and Doly [29]. Basically, 20 ml of culture was grown overnight and the bacteria were harvested by centrifugation for 5 minutes at 5000 rpm using the JA-20 rotor (Beckman Avanti J-20 centrifuge). Next 0.8 ml ice-cold Doly 1 solution (1 M tris, 0.5 M EDTA, 20% glucose), 1.6 ml Doly 2 solution (2 N NaOH, 10% SDS), and 1.2 ml of 3 M sodium acetate were added to the bacterial pellet. After the pellet was dissolved, it was centrifuged at 8000 rpm for 10 minutes. The supernatant was mixed with an equal amount of isopropanol. The mixture was incubated at 4°C for 30 minutes. The pellet gathered by centrifugation at 14,000 rpm for 5 minutes was dissolved in 0.45 ml Doly 4 solution (0.1 M sodium acetate, 50 mM tris HCl). Then 1.1 ml EtOH (99.9%) was added and the mixture was left in -20°C for 30 minutes. The precipitate was

gathered by centrifuging the mixture at 14,000 rpm (16,000 × g) in a microcentrifuge for 5 minutes and then dissolved in 20 µl TE.

Transformation of the pO157::Tn801 to O157:H⁻ (rif^r, ΔpO157).

Electroporation was used to introduce the Tn801-marked plasmid to O157:H⁻ (rif^r, ΔpO157) and the mutants were selected by growing the transformants on TSA plates containing carbenicillin (250ug/ml) and rifampin (250ug/ml) at 37°C. Electroporation was performed in Gene Pulser cuvettes (0.1cm electrode gap) at 1.8 kvolt and 200 Ω with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) by following the instructions of the manufacture.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblot analysis. SDS-PAGE was performed in a Bio-Rad minicell apparatus according to the procedure described by Laemmli [30]. The 13% polyacrylamide gels were Coomassie Blue stained or silver stained [31]. Alternatively, the proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were soaked in TTBS+3% BSA at 4°C overnight. Then membranes were probed with rabbit anti-BFPA (bundle-forming pili) antibody diluted to 1:7000 and detected with anti-rabbit IgG secondary antibody diluted to 1:5000.

2.4.2. Results

Plasmid analysis.

Tn801 was inserted into pO157 and the pO157::Tn801 was successfully isolated from the conjugant O157:H⁻ (pO157::Tn801) grown in LB with carbenicillin (Figure 2.6.). I intended to transform pO157::Tn801 by electroporation into recipient O157:H⁻ (rif^r, ΔpO157). However, despite making numerous

modifications to the electroporation conditions, I failed to transform pO157::Tn801 into the recipient. This may be because O157:H⁻ (rif^r, ΔpO157) is a poor recipient and pO157 is too big to be transferred. However, I did other experiments to prove that the O157:H⁻ (rif^r, ΔpO157) was derived from O157:H⁻. The plasmid-cured derivative of *E. coli* O157:H⁻ produced the same amount of Stx (Figure 2.7.), and agglutinated to the same extent in ammonium sulfate as the wild-type strain from which it was derived. Therefore, by these criteria, the plasmid-cured *E. coli* O157:H⁻ strain only differed from the wild-type strain with respect to the absence of pO157. The different binding efficiency between the parental strain and the plasmidless strain is not due to differences in hydrophobicity since the salt aggregation test showed that the surface hydrophobicity of the two strains was the same.

Pili analysis.

Transmission electron microscopy revealed that all the EHEC strains, including *E. coli* O157:H⁻ , *E. coli* O157:H⁻ (rif^r , ΔpO157), and all 10 clinical isolates of *E. coli* O157:H7, produced slender filamentous surface appendages resembling pili when grown in glucose-free TSB (Figure 2.5.). When these strains were grown in TSB or LB, little or no pili were observed in the ammonium sulfate precipitates. Pili magnified at 90,666 × were calculated to be approximately 0.5 nm in diameter.

Figure 2.6. 1% agarose gel of PCR product. The 1,551-bp amplification products were analyzed by electrophoresis and stained by ethidium bromide.

From left to right:

1 kb ladder standard

PCR product of plasmid prepared from O157:H$^-$ (pO157::Tn801)

PCR product of plasmid prepared from O157:H$^-$ (rif^r, ΔpO157)

PCR product of plasmid prepared from O157:H$^-$

PCR product of plasmid prepared from O157:H7

PCR product of plasmid prepared from O157:H$^-$ (pO157::Tn801)

PCR product of plasmid prepared from O157:H$^-$ (pO157::Tn801)

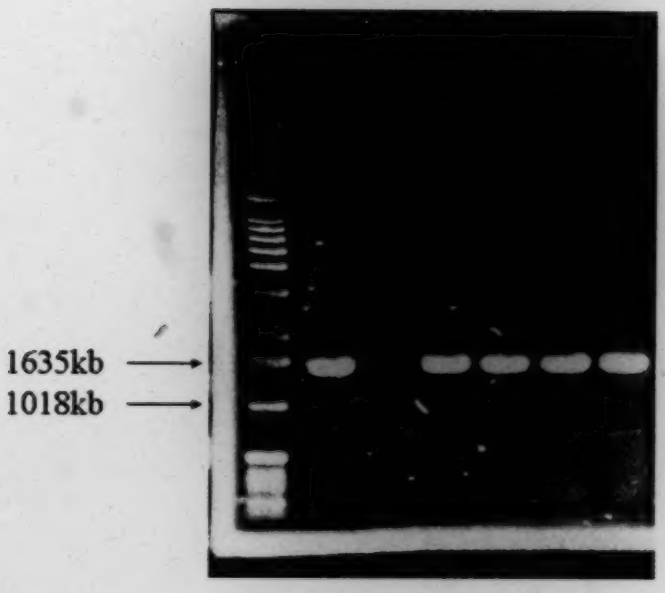
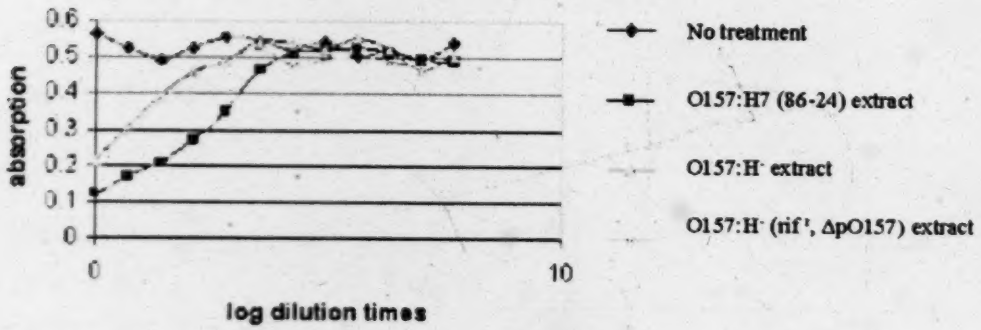

1635kb
1018kb

Figure 2.7. Vero assay comparing Stx production by O157:H⁻ and O157:H⁻ (rif^r, ΔpO157). One ml of whole bacterial cells was treated with 0.1mg/ml polymyxin B sulfate for 30 min. at 37°C to release the toxin. Bacteria were removed by centrifugation at 16,000 × g for 5 min. in 1.5 ml micro-centrifuge tubes using an Eppendorf model 5415C centrifuge. The resulting supernatant solution was filter-sterilized through a 0.2 μm filter. 1:5 serial dilutions of the extracts were prepared and added to confluent Vero cell monolayers in 96-well microtiter plates. After incubation for 48 hours, the Vero cells were fixed with methanol and stained with Geimsa stain. The results were recorded using a microtiter plate reader set at a wavelength of 620 nm and LD_{50} was calculated as described by Armstrong, G. D. *et al.* [11].

Vero assay (compare verocytoxity between O157:H7, O157:H-, and O157:H- (rif ^{r}, ΔpO157)



The ammonium sulfate precipitation did not react with anti-BFP antibody, which indicate that has no relation with BFP produced by EPEC (Figure 2.9.)

Although I initially felt I may have identified another fimbrial-like adhesin in *E. coli* O157:H$^-$ grown in glucose-free TSB, a recent report by Gaynor *et al.* prompted us to examine the origin of the filamentous structures more carefully [16]. Sterile glucose-free TSB incubated overnight also produced filamentous structures, resembling those seen in the O157 culture. This led us to consider the possibility that the pili I discovered may also be an artifact (Figure 2.5.). I also attempted to purify the pili from *E. coli* O157:H$^-$ grown in glucose-free TSB using ammonium sulfate precipitation and CsCl density gradient centrifugation as described previously [12]. This produced three bands and a pellet in the CsCl gradients. SDS polyacrylamide gel electrophoresis analysis revealed that the top band (density approximately 1.2g/cm^3) contained mainly four proteins with molecular weights of 85kDa, 26kDa, 15kDa, and 13kDa (Figure 2.8.). Although the 15kDa and the 13kDa bands may have represented a pilus structural subunit, microsequence analysis demonstrated that the 15kDa protein was homologous to host factor I for bacteriophage Q beta replication, a growth-related protein in *E. coli*. The 13kDa protein gave a signal so weak that it could not be analysed. Microsequence analysis of the 26 kDa SDS-PAGE band did not produce any clear indication of its likely identity since it gave us a weak and ambiguous signal. It may be caused by N-terminal block of the protein and by underlying co-migrating proteins.

Figure 2.8. 13% SDS-PAGE gel of ammonium sulfate precipitated proteins and top band of CsCl gradient from *E. coli* O157:H⁻. Lane1. Low molecular weight standard, Lane 2. Crude ammonium sulfate precipitated proteins from *E. coli* O157:H⁻, Lane 3. Top band of CsCl gradient (density approximately 1.2g/cm^3) removed after processing of the crude extract.

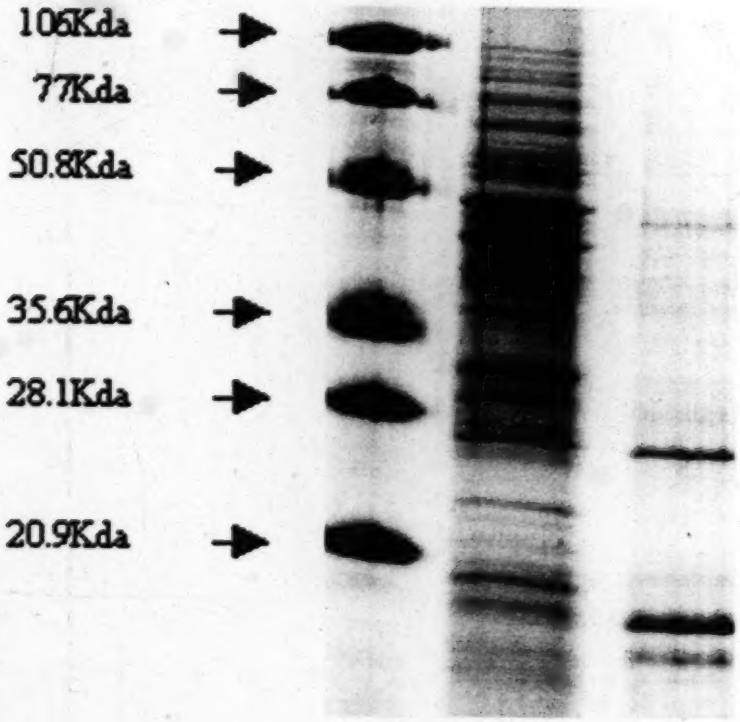
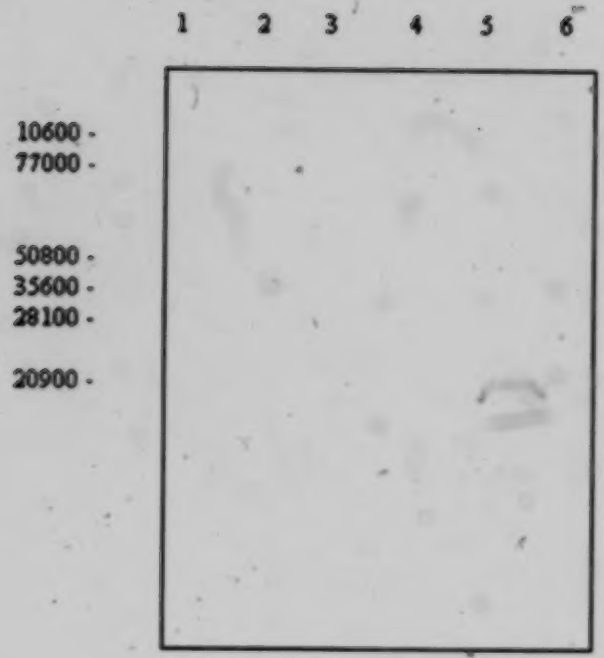

106Kda
77Kda
50.8Kda
35.6Kda
28.1Kda
20.9Kda

Figure 2.9. Western-blot of ammonium sulfate precipitation probed with rabbit anti-BFPA (bundle-forming pili) antibody diluted to 1:7000 and detected with anti-rabbit IgG secondary antibody. Lane 1: O157:H$^-$, lane 2: O157:H$^-$ (rif^r, ΔpO157), lane 3: O157:H7 (86-24), lane 4: O157:H7 (CVD 451), lane 5: EPEC E2348/69, lane 6: EPEC JPN15. All EHEC strains, including lane 1-4, did not react with anti-BFP A antibody.


1
2
3
4
5
6
10600 -
77000 -
50800 -
35600 -
28100 -
20900 -

Moreover, TEM analysis revealed only one fimbrial morphotype in the CsCl-purified sample. Therefore, My inability to obtain conclusive evidence for the presence of pilin in the CsCl preparation of the filamentous structures and the appearance of only one pili morphotype made us believe that these were probably the same artifactual structures which appeared in sterile glucose-free TSB incubated overnight at 37°C. However, I cannot exclude the possibility that O157:H$^-$ and its plasmid-cured strain do produce pili and further investigation is needed.

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CHAPTER 3

General Discussion

I found that the binding of the plasmid-cured EHEC strain to HEp-2 and Intestine 407 cells was significantly decreased in comparison to that of the wild-type strain, indicating that pO157 likely plays a role in EHEC binding to these tissue culture cells. In order to prove further the function of pO157 in the binding of *E. coli* O157:H$^-$ to tissue culture cells, I tried but failed to reintroduce pO157 into O157:H$^-$ (rif ^{r}, ΔpO157). However, other experiments were carefully done to prove that the O157:H$^-$ (rif ^{r}, ΔpO157) was derived from wild-type O157:H$^-$. The plasmid-cured derivative of *E. coli* O157:H$^-$ produced the same amount of Stx, and agglutinated to the same extent in ammonium sulfate as the wild-type strain from which it was derived. Therefore, by these criteria, the plasmid-cured *E. coli* O157:H$^-$ strain differed from the wild-type strain only with respect to the absence of pO157. The difference in binding between the parental strain and the plasmidless strain was not due to the differences in hydrophobicity since the salt aggregation test demonstrated that the surface hydrophobicity of the two strains was the same.

Many studies have used Intestine 407 cells to represent the cell line derived from the intestine. However, Intestine 407 was established via HeLa cell contamination and contains Hela cell markers [1]. Since Caco-2 and T84 cells are derived from human colon cells, I also performed binding assays using these two cell lines. However, no difference between the plasmidless derivative and the parental strain was seen. Overall, my results suggest that the presence of the plasmid affects the

binding of O157:H⁻ to HEp-2 and Intestine 407 cells, but does not affect binding to Caco-2 and T84 cells.

The sequence of pO157 does not reveal any potential adhesin genes on the plasmid [2]. So pO157 may regulate adhesins expressed by genes on the chromosome. Twelve potential adhesins have been identified in the O157:H7 genome [3] and there may be more genes contributing to the adherence of these organisms to host cells. None of these adhesins, though, can explain the complexity of O157 adhesion in all circumstances. I have shown different binding results of the organisms in different conditions and found the organisms expressed different adhesins in different situations. In addition to colonizing in the human intestine, the O157 strains can also live in the digestive systems of animals, especially cattle [4]. The digestive system of cattle is very different from that of humans. So EHEC may have to use different adherent mechanisms in different hosts. The over twelve potential adhesins suggested by genome sequencing indicate that there may be redundancy of O157 adhesins and one or more of them may be expressed in different situations.

So far, I can say that pO157 is important in mediating the binding of O157:H⁻ to HEp-2 and Intestine 407 cells. The O157 strains need to adhere first in human intestinal cells but my experiments did not suggest that pO157 regulate the adherence in this situation as shown in the Caco-2 and T84 binding assay. All O157 strains are pathogenic and all of them have the pO157 [4]. So pO157 is associated with pathogenicity. Although HEp-2 and Intestine 407 cells were not derived from human intestine cells, they may be similar to the bovine intestine

cells. Plasmid O157 may be important for binding to bovine intestine cell lines. There are not bovine intestine cell lines available. I tried Madin-Darby bovine kidney (MDBK) cell line in my binding assay using the plasmid-cured strain and the parental strain but did not get consistent positive control. A streptomycin-treated mouse model was used to investigate the colonization function of pO157. The findings indicated that O157:H7 cured of the pO157 could not compete with the wild-type in colonizing the mice [5]. So pO157 seems to regulate some stage in colonization, i.e. attachment, nutrient acquisition, or resistant to antimicrobial factors, in this model. The result that pO157 does not regulate the binding of O157:H$^-$ to Caco-2 and T84 cells does not rule out the possibility that pO157 regulates adherence *in vivo*. Since *in vitro* tissue culture cells gave an unclear picture of the function of pO157, other *in vitro* or *in vivo* methods should be used in order to identify the role of pO157 in pathogenesis. *In vitro* organ culture (IVOC) model [6] and *in vivo* animal model are better ways to explore the function of pO157. The streptomycin-treated mouse model, one-day-old chicken model [7], neonatal calf model [8], and gnotobiotic pig model [9, 10] can be used in studying the colonization of O157 strains. The streptomycin-treated mouse model would be the most practical model to study the colonization of pO157 *in vivo* in the future because the mice are small, easy to handle and cheaper than most other animals.

The reason why different labs have reported different binding results for O157:H7 and its plasmid-cured derivatives may partially be due to the different strains used (O157:H7 strain 933, strain 932, strain 7785), different culture media (Mueller-

Hinton broth, penassay broth), different phases of growth (culture time ranging from 16-48 hours), different binding periods used for the assays (ranging from 1-5 hours), or different inoculum concentrations (10^7-10^8).

I performed binding assays using different tissue culture cell lines and obtained varied results, indicating the importance of using more than one type of cell line in these assays. I saw pili-like structures when both plasmid-cured strain and parental strain were grown in glucose-free TSB but not in TSB or LB. Pili, which were isolated from all EHEC O157:H$^-$, and O157:H7 strains had similar protein profiles. Besides, the pili produced by O157:H$^-$ and O157:H7 had similar structure confirmed by EM. However, I found the similar pili-like structures in sterile glucose-free TSB, which suggested the pili I saw were artifacts. It shows how important it is to have the negative control in studying pili, which most groups did not pay attention before.

There is also another shortcoming of my experiment. That is, I did not reintroduce the pO157 into the plasmid-cured strain to reinstall the wild-type phenotype. This procedure is technically difficult since pO157 is large in size and O157:H$^-$ (rif^r, ΔpO157) is not a good recipient. My experiments could get around this difficulty by subcloning pieces of pO157 and reintroducing the pieces into the recipient. This can be done by cutting the pO157 with restriction enzymes, subcloning the pieces into vectors, and then introducing the vectors into the plasmidless strain by electroporation. If one or two pieces reinstall the recipient with the wild-type adherence ability to HEp-2 or Intestine 407, I can not only confirm that the pO157 regulates an adhesin or adhesins, but also can point out where the regulatory site

or sites are in pO157. In fact, Tatsuno *et al.* introduced *toxB*, a gene in pO157, to the plasmid-cured strain of O157:H7 (O157Sakai) and restored the full adherence capacity to Caco-2 cells compared with the parental strain [11]. Their experiment suggested that *toxB* contribute to the adherence through enhancement of the type III secretion system. Plamid O157 seems to mediate adherence to Caco-2 cells in their experiment. This may be due to the different strains we used. It again shows how complex the mechanisms of the plasmid O157 in adhering to the tissue culture cells.

Identifying the regulatory site or sites on pO157 and identifying the potential adhesins will lead to effective ways of preventing these organisms from interacting with host cells.

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